PATENT
454313-2200

IN THE UNITED STATES PATENT AND TERADEMARK

APPLICATION FOR LETTERS PATENT

TITLE: CANINE HERPESVIRUS BASED RECOMBINANT LIVE VACCINE, IN PARTICULAR AGAINST CANINE DISTEMPER, RABIES OR THE PARAINFLUENZA 2 VIRUS

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CLAIMS: 16 (2 independent)

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Recombinant live vaccine based on canine herpesvirus, in particular against Carré's disease, rabies or the parainfluenza virus type 2.

The present invention relates to vaccines, preferably for dogs, produced from recombinant canine herpesviruses, and to the methods for obtaining and preparing these recombinant viruses. The present invention relates more especially to recombinant canine herpesviruses comprising an expression cassette for one or more foreign gene(s).

Canine herpesvirosis is caused by the canine herpesvirus (CHV). The canine herpesvirus (CHV) is classified in the *Alphaherpesvirinae* family. This herpesvirus is a major pathogen for neonatal puppies. Canine herpesvirosis manifests itself chiefly in a haemorrhagic disease in puppies, and in a benign disease of the upper respiratory apparatus in adult dogs. There are at present no vaccines for protecting puppies against canine herpesvirosis.

Moreover, domestic dogs are exposed to numerous other diseases, and the development of a vaccinal vector capable of expressing different antigens of canine pathogenic agents would enable the efficacy of vaccination programmes to be simplified and improved, especially for puppies in breeding kennels. Among pathogenic agents of importance for dogs, the Carré's disease virus, the Rubarth's hepatitis virus, the rabies virus, the canine parvovirosis virus, the canine coronavirus, the parainfluenza virus type 2, *Bordetella bronchiseptica*, *Borrelia burgdorferi*, *Leptospira spp.* and *Leishmania infantum* may be mentioned.

Little is known about the CHV virus genome. The genomic organization of this virus was published only recently (Rémond M. *et al.* J. Gen. Virol. 1996. **77**. 37-48), and the genes for the three major glycoproteins gB, gC and gD, as well as a gene designated CHV ORF2, have been described (K. Limbach *et al.* J. Gen. Virol. 1994. **75**. 2029-2039).

Following their work on CHV, the inventors have succeeded in determining several regions which are non-essential for replication *in vitro*, which have proved useful for the construction of recombinant CHV viruses. The inventors are hence in a position to put forward for the first time the CHV virus as a vaccination vector for dogs. It was found that the vaccinal vectors according to the invention had particular advantages for the vaccination of dogs. In effect, the canine herpesvirus is very species-specific and possesses a large genome containing several potential insertion sites and permitting the simultaneous insertion of several expression cassettes for foreign genes. This affords the possibility of vaccinating dogs at the same time against different canine pathogenic agents using a single recombinant virus.

The main objective of the invention is to provide a vaccinal vector permitting the expression of immunogens of canine pathogens for the purpose of protecting dogs against the main canine infectious diseases.

Another objective of the invention is to provide such a vector permitting the vaccination of dogs, and especially puppies having maternal antibodies, via the mucosal, in particular the oral, nasal or conjunctival, route.

Yet another objective of the invention is to provide such a vector which permits vaccination at the same time against herpesvirosis in puppies.

Hence the subject of the present invention is a recombinant live vaccine using as vector a canine herpesvirus comprising and expressing at least one nucleotide sequence coding for a polypeptide, this sequence being inserted into a site which is non-essential for replication *in vitro*.

The inventors have isolated and analysed a genomic fragment of the CHV virus, on which they have characterized 5 open reading frames (ORF1 to ORF5), among which two (ORF3 and ORF5) have proved to be non-

essential for replication *in vitro*. Moreover, the inventors have found that other regions of the CHV genome could also be used to insert foreign genes. These insertion sites are: thymidine kinase gene (CHV TK ORF) (Rémond M. *et al*. Virus Research. 1995. **39**. 341-354.) and sequence situated between the CHV ORF19 and the CHV ORF22 (Rémond M. *et al*. J. Gen. Virol. 1996. **76**. 37-48). These sites are described more precisely in the examples of the present invention.

Preferably, the inserted sequence codes for an antigenic polypeptide, and preferentially for an antigenic polypeptide of a canine pathogenic agent. It is also possible to insert the sequences coding for immunomodulatory proteins such as cytokines. According to an advantageous variant, it is possible to use in combination a sequence coding for a cytokine, or the like, and a sequence coding for an antigen. If need be, several cytokine sequences can be used in combination with one another, optionally in combination with one or more sequences coding for antigens.

The insertion into the sites is carried out by simple insertion (without deletion), or after partial or total deletion of the ORF or ORFs used as insertion sites.

As a parent virus for the construction of recombinant CHV viruses, it is possible to use, in particular, the CHV strain F205 which was isolated by L. Carmichael (Proc. Soc. Exp. Biol. Med. 1965. **120**. 644-650).

For the expression of foreign genes inserted into the CHV genome according to the present invention, it will be preferable to use a strong eukaryotic promoter such as, preferentially, a cytomegalovirus (CMV) immediate-early (IE) promoter. CMV IE promoter is understood to mean, in particular, a fragment such as is given in the examples, as well as the subfragments thereof retaining the same promoter activity. The CMV IE promoter can be the human (HCMV IE) promoter or the murine (MCMV IE) promoter, or alternatively a CMV IE

promoter of another origin, for example rat, guinea pig or porcine CMV.

At least two nucleotide sequences may be inserted into one site under the control of different promoters. The latter may be, in particular, CMV IE promoters of different origins.

According to an advantageous development of the invention, another promoter is used in combination with the CMV IE promoter in such a way that the two promoters have their 5' ends adjacent and that the transcriptions initiated from these two promoters take place in opposite directions. This particular arrangement enables two nucleotide sequences to be inserted into the same site, one under the control of the CMV IE promoter and the other under that of the promoter used in combination with it. This construction is noteworthy from the fact that the presence of the CMV IE promoter, and in particular of its enhancer portion, can activate the transcription induced by the promoter used in combination. As a promoter used in combination, there may be mentioned, for example, a CMV promoter of different species from the first promoter. It is also possible to envisage other promoters, such as the Marek's disease virus (MDV) RNA1.8 promoter (G. Bradley *et al*. J. Virol. 1989. **63**. 2534-2542).

The nucleotide sequence inserted into the CHV vector in order to be expressed can be any sequence coding for an antigenic polypeptide of a canine pathogenic agent capable, when expressed under the favourable conditions obtained by the invention, of bringing about an immunization leading to an effective protection of the vaccinated animal against the pathogenic agent. The nucleotide sequences coding for the antigens of interest for a given disease, in particular the viral, bacterial or parasitic diseases mentioned above, may hence be inserted under the conditions described by the present invention.

The typical case of the invention is the insertion of at least one nucleotide sequence coding

appropriately for a polypeptide of the Carré's disease virus (canine distemper virus = CDV), and preferably for the CDV polypeptide HA (Sidhu M. *et al.*, Virology. 1993. **193**. 66-72) or for the CDV polypeptide F (Barrett T. *et al.* Virus Research. 1987. **8**. 373-386). It is also possible to insert both of these genes together into the CHV vector. A recombinant live vaccine bringing about protection against Carré's disease is thereby obtained.

Other preferred cases of the invention are the insertion of nucleotide sequences coding for antigens or fragments of antigens of the rabies virus, especially the G gene (Patents FR-A-2,515,685 and EP-A-162,757), of the canine parvovirosis virus (VP2 gene) (Parrish C. *et al.* J. Virol. 1991. **65**. 6544-6552) or of the parainfluenza virus type 2 (HA and/or F genes). It is also possible to insert sequences coding for *Borrelia burgdorferi* antigens, especially the genes coding for the OspA and OspB antigens (Bergström S. *et al.* Mol. Microbiol. 1989. **3**. 479-486).

A typical case of the invention is a vaccine comprising a nucleotide sequence coding for an antigen of the Carré's disease virus under the control of CMV IE, and a nucleotide sequence coding for an antigen of another canine viral disease, in particular the ones mentioned above, under the control of the other promoter.

Naturally, the heterologous sequences and their associated promoters may be inserted more conventionally in tandem into the insertion locus, that is to say according to the same transcription direction.

The expression of several heterologous genes inserted into the insertion locus can also be possible by insertion of a sequence known as an "IRES" (internal ribosome entry site) originating, in particular, from a picornavirus such as the swine vesicular disease virus (SVDV; B.-F. Chen *et al.*, *J. Virology*, 1993, **67**, 2142-2148), the encephalomyocarditis virus (EMCV; R.J.

Kaufman *et al.*, *Nucleic Acids Research*, 1991, **19**, 4485-4490) or the aphthous fever virus (FMDV; N. Luz and E. Beck, *J. Virology*, 1991, **65**, 6486-6494), or alternatively of another origin. The content of these three papers is incorporated by reference. The cassette for expression of two genes would hence have the following minimum structure: promoter - gene 1 - IRES - gene 2 - polyadenylation signal. The recombinant live vaccine according to the invention may hence comprise, inserted into the insertion locus, an expression cassette comprising in succession a promoter, two or more genes separated in pairs by an IRES, and a polyadenylation signal.

In addition to the insertion into the locus according to the invention, it is possible to carry out one or more other insertions, one or more mutations or one or more deletions elsewhere in the genome. In all cases, insertion into a locus other than the one described in the invention enables other genes to be expressed.

The use of the recombinant viruses according to the invention enables dogs to be protected against one or more of the diseases mentioned above, and at the same time against canine herpesvirosis.

The subject of the present invention is also a polyvalent vaccine formula comprising, as a mixture or to be mixed, at least two recombinant live vaccines as defined above, these vaccines comprising different inserted sequences isolated, in particular, from different pathogens. These vaccine formulae contain dosages and/or vehicles which are suited to the administration route.

The subject of the present invention is also CHV viruses modified in at least one of the sites indicated.

Its subject is also a method of vaccination, especially of dogs, in which an effective amount of a vaccine as defined above is administered via any parenteral or mucosal route, but preferably via the

mucosal, in particular the oral and/or nasal, route. The vaccinal dose will preferably be between 10^2 CCID50 and 10^7 CCID50. Preferably, the dose for the parenteral route will be between 10^4 CCID50 and 10^7 CCID50, and for the oral and/or nasal route, between 10^2 CCID50 and 10^5 CCID50. As defined, the vaccine is effective in general after a single administration via the oral and/or nasal route. However, repeated administrations may be necessary.

The subject of the present invention is also the DNA fragments comprising all or part of the sequence defined by positions 1 to 6216 on SEQ ID No. 1 (Figure No. 1), in particular all or part of the ORF3 site defined and/or of the flanking sequences located upstream and downstream of this site, which fragments will be useful as flanking arms for the techniques of homologous recombination with the genome of the CHV virus chosen as parent virus. Naturally, the invention also relates to the variants of these fragments which correspond to the equivalent sequences of the other strains of CHV. The expert is entirely free to choose the regions serving as flanking arms in connection with the type of insertion (with or without deletion) or of deletion (partial or total) chosen. Generally speaking, the flanking arms may thus have from 100 to 800 base pairs, but can be larger if necessary.

A further subject of the invention is a method of preparation of the vectors and vaccines according to the invention, as emerges from the description of the vaccines, by insertion of genes of interest into the insertion site.

The invention will now be described in greater detail by means of non-limiting examples of implementation, taken with reference to the drawing, wherein:

Figure 1: Sequence of the CHV region (6216 base pairs) and translation of the different open reading frames (ORFs) present in this sequence (ORF1 to ORF5).

Figure 2: Plasmid pPB200 (donor plasmid for the insertion of expression cassettes into the CHV ORF3 site).

Figure 3: Construction of the plasmid pPB202 (donor plasmid for the insertion of expression cassettes into the CHV ORF5 site).

Figure 4: Construction of the plasmid pPB204 (donor plasmid for the insertion of expression cassettes into the CHV TK site).

Figure 5: Construction of the plasmid pPB206 (donor plasmid for the insertion of expression cassettes into the site situated between the CHV ORF19 and CHV ORF22 genes).

Figure 6: Construction of the plasmid pPB208 (expression cassette for the CDV HA gene).

Figure 7: Construction of the plasmid pPB210 (expression cassette for the CDV F gene).

Figure 8: Construction of the plasmid pPB213 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF3 site).

Figure 9: Construction of the plasmid pPB214 (donor plasmid for the insertion of the cassette for the expression of the CDV F gene into the CHV ORF3 site).

Figure 10: Plasmid pPB200'

Figure 11: Construction of the plasmid pPB212 (cassette for the expression of the rabies virus G gene).

Figure 12: Construction of the plasmid pPB125 (donor plasmid for the insertion of the cassette for the expression of the rabies virus G gene into the CHV ORF3 site).

Figure 13: Construction of the plasmid pPB216 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF5 site).

Figure 14: Construction of the plasmid pPB217 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the CHV TK site).

Figure 15: Construction of the plasmid pPB218 (donor plasmid for the insertion of the cassette for the expression of the CDV HA gene into the site situated between the CHV ORF19 and CHV ORF22 genes).

SEQ ID sequence listing for the constructions in the insertion sites of the CHV vector:

Sub. B1

~~**SEQ ID No. 1** Complete sequence of the CHV ORF1 → ORF5 region depicted in Figure 1~~

SEQ ID No. 2 ORF1 amino acid sequence of Figure 1

SEQ ID No. 3 ORF2 amino acid sequence of Figure 1

SEQ ID No. 4 ORF3 amino acid sequence of Figure 1

SEQ ID No. 5 ORF4 amino acid sequence of Figure 1

SEQ ID No. 6 (Partial) ORF5 amino acid sequence of Figure 1

SEQ ID No. 7 Oligonucleotide JCA070

SEQ ID No. 8 Oligonucleotide JCA071

SEQ ID No. 9 Oligonucleotide JCA072

SEQ ID No. 10 Oligonucleotide JCA073

SEQ ID No. 11 Oligonucleotide JCA074

SEQ ID No. 12 Oligonucleotide JCA075

SEQ ID No. 13 Oligonucleotide JCA076

SEQ ID No. 14 Oligonucleotide JCA077

SEQ ID No. 15 Oligonucleotide JCA078

SEQ ID No. 16 Oligonucleotide JCA079

SEQ ID No. 17 Oligonucleotide JCA080

SEQ ID No. 18 Oligonucleotide JCA081

SEQ ID No. 19 Oligonucleotide JCA082

SEQ ID No. 20 Oligonucleotide JCA083

SEQ ID No. 21 Oligonucleotide JCA084
SEQ ID No. 22 Oligonucleotide JCA085
SEQ ID No. 23 Oligonucleotide PB088
SEQ ID No. 24 Oligonucleotide PB089
SEQ ID No. 25 Oligonucleotide JCA086
SEQ ID No. 26 Oligonucleotide JCA087
SEQ ID No. 27 Oligonucleotide JCA088
SEQ ID No. 28 Oligonucleotide JCA089
SEQ ID No. 29 Oligonucleotide JCA090
SEQ ID No. 30 Oligonucleotide JCA091

EXAMPLES

All the constructions of plasmids were carried out using the standard techniques of molecular biology described by Sambrook J. *et al.* *(Molecular Cloning: A Laboratory Manual.* 2nd Edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York. 1989). All the restriction fragments used for the present invention were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

The virus used as parent virus is the canine herpesvirus strain F205 (also known as the Carmichael strain). This strain was obtained from Dr. L. Carmichael (Cornell University, NY), who isolated it and described its biological characteristics (Proc. Soc. Exp. Biol. Med. 1965. **120**. 644-650). The conditions of culture of this virus are as follows: MDCK (Madin-Darby canine kidney ATCC CCL34) cells cultured in Eagle's minimum essential medium (MEM medium) are inoculated with the CHV strain F205 using a multiplicity of infection of 1. The infected cells are then incubated at 37°C for approximately 36 hours until a complete cytopathic effect is seen.

Example 1: Extraction of canine herpesvirus DNA

After culture, the supernatant and the lysed cells are harvested, and the whole of the viral suspension is centrifuged at 1000 g for 10 minutes at +4°C to remove cell debris. The viral particles are

then harvested by ultracentrifugation at 400,000 g for 1 hour at +4°C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral suspension is treated with proteinase K (100 µg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a phenol/chloroform mixture and thereafter precipitated with 2 volumes of absolute ethanol. After one night at -20°C, the precipitated DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water.

Example 2: Cloning and characterization of the CHV ORF1 - ORF5 region

The purified genomic DNA of the CHV virus strain F205 was digested with the restriction enzymes *Sca*I and *Xho*I, and the approximately 6200-bp ScaI-XhoI fragment was cloned into the vector pBlueScript SKII+ (Stratagene Ref. 212205), previously digested with *Sca*I and *Xho*I, to give the plasmid pPB154.

The XhoI-ScaI fragment cloned into plasmid pPB154 was sequenced completely on both strands to generate the 6216-bp sequence of Figure 1 (SEQ ID No. 1).

Several open reading frames larger than 65 amino acids in size were identified on this sequence (Figure 1):

The first reading frame (ORF1) (positions 1353 - 157) occurs on the complementary strand and codes for a polypeptide of 398 amino acids (SEQ ID No. 2).

The second reading frame (ORF2) (positions 1708 - 2970) codes for a polypeptide of 420 amino acids (SEQ ID No. 3).

The third reading frame (ORF3) (positions 3040 - 4242) codes for a polypeptide of 400 amino acids (SEQ ID No. 4).

The fourth reading frame (ORF4) (positions 4374 - 5753) codes for a polypeptide of 459 amino acids (SEQ ID No. 5).

The fifth reading frame (ORF5) (positions 5872 - 6216) is incomplete and codes for a truncated protein of 115 amino acids (SEQ ID No. 6).

The different open reading frames are collated in the table below:

Open reading frame	Beginning - End (positions in Figure 1)	Size in amino acids
ORF 1	1353 - 157	398 aa
ORF 2	1708 - 2970	420 aa
ORF 3	3040 - 4242	400 aa
ORF 4	4374 - 5753	459 aa
ORF 5	5872 - 6216	115 aa

Example 3: Construction of plasmid pPB200 for the insertion of expression cassettes into the CHV ORF3 site (Figure 2)

Plasmid pPB154 (9121 bp) (Example 2) was digested with *Hin*dIII and *Spe*I to isolate the 620-bp HindIII-SpeI fragment (fragment A). Plasmid pPB154 was digested with *Eco*RI and *Spe*I to isolate the 659-bp EcoRI-SpeI fragment (fragment B). The fragments A and B were ligated together with the vector pGEM4Z (Promega Ref. P2161), previously digested with *Eco*RI and *Hin*dIII, to give the plasmid pPB199 (4096 bp). Plasmid pPB199 was then digested with *Spe*I, treated with alkaline phosphatase and then ligated with the multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA070 (33 mer) (SEQ ID No. 7)

5' CTAGTCCAGCAAGGTGGATCGATATCGGGCCCA 3'

JCA071 (33 mer) (SEQ ID No. 8)

5' CTAGTGGGCCCGATATCGATCCACCTTGCTGGA 3'

to give plasmid pPB200 (4129 bp).

Example 4: Construction of plasmid pPB202 for the insertion of expression cassettes into the CHV ORF5 site (Figure 3)

The sequence of the CHV ORF5 gene was published recently (Limbach K. *et al.* J. Gen. Virol. 1994. 75. 2029-2039). A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:

JCA072 (22 mer) (SEQ ID No. 9)

5'CAGCTTTATGTTTTTATTGTTC 3'

JCA073 (29 mer) (SEQ ID No. 10)

5'AAAGAATTCTACAACTGTTTAATAAAGAC 3'

to obtain a 751-bp PCR fragment containing the complete CHV ORF2 gene. This fragment was digested with *BGlII* and *EcoRI* to isolate a 709-bp BglII-EcoRI fragment. This fragment was ligated with the vector pGEM4Z (Promega Ref. P2161), previously digested with *EcoRI* and *BamHI*, to give the plasmid pPB201 (3559 bp). Plasmid pPB201 was then digested with *ScaI* and *PvuII* and thereafter ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA074 (36 mer) (SEQ ID No. 11)

5'ACTCCAGCTACATGGGATATCGGGCCCATCGATCAG 3'

JCA075 (36 mer) (SEQ ID No. 12)

5'CTGATCGATGGGCCCGATATCCCATGTAGCTGGAGT 3'

to give plasmid pPB202 (3395 bp).

Example 5: Construction of plasmid pPB204 for the insertion of expression cassettes into the CHV TK site (Figure 4)

The sequence of the CHV thymidine kinase (TK) gene was published recently (Rémond M. *et al.* Virus Research. 1995. 39. 341-354). A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:

JCA076 (35 mer) (SEQ ID No. 13)

5'AGCGTTAACCTCAAAAGCCAAATTTACACTTCCCG 3'

JCA077 (38 mer) (SEQ ID No. 14)
5'CCCAAGCTTTTCTAAAGCCCATTTATAAATAATAAATG 3'
to obtain a 1030-bp PCR fragment containing the thymidine kinase (TK) gene. This fragment was digested with *Hpa*I and *Hind*III to isolate a 1019-bp HpaI-HindIII fragment. This fragment was ligated with the vector pSP73 (Promega Ref. P2221), previously digested with *EcoRV* and *Hind*III, to give the plasmid pPB203 (3423 bp).

Plasmid pPB203 was then digested with *EcoR*I and *Sty*I and thereafter ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:
JCA078 (36 mer) (SEQ ID No. 15)
5'AATTCCCAGCTACATGGGATATCGGGCCCATCGATC 3'
JCA079 (36 mer) (SEQ ID No. 16)
5'CAAGGATCGATGGGCCCGATATCCCATGTAGCTGGG 3'
to give plasmid pPB204 (3399 bp).

Example 6: Construction of plasmid pPB206 for the insertion of expression cassettes into the site situated between the CHV ORF19 and ORF22 genes (Figure 5)

The sequence of the intergenic region corresponding to the natural deletion of the genes coding for the large subunit ("RR1" gene) and for the small subunit ("RR2" gene) of ribonucleotide reductase was published recently (Rémond M. *et al.* J. Gen. Virol. 1996, **77**. 37-48). According to the nomenclature used by Rémond *et al.*, the deletion of these two genes occurs between the open reading frames designated CHV "orf19" and CHV "orf22" [designated herein ORF19 and ORF 22, respectively]. A PCR reaction was carried out with the genomic DNA of the CHV virus strain F205 (Example 1) and with the following oligonucleotides:
JCA080 (36 mer) (SEQ ID No. 17)
5'GGAGATCTAGTAAATTAAATAGTAATTCATTTAATG 3'
JCA081 (33 mer) (SEQ ID No. 18)
5'CAGTCGCGAAGATGAAAATAAAATCCATCGAAG 3'

to obtain a 720-bp PCR fragment containing the intergenic region corresponding to the natural deletion of the CHV ORF19 and ORF22 genes. This fragment was digested with *Spe*I and *Nru*I to isolate a 709-bp SpeI-NruI fragment. This fragment was ligated with the vector pGEM4Z (Promega Ref. P2161), previously digested with *Sma*I and *Xba*I, to give the plasmid pPB205 (3572 bp). Plasmid pPB205 was then digested with *Mfe*I and thereafter partially digested with *Ssp*I in order to isolate the 3512-bp MfeI-SspI fragment. This fragment was then ligated with a multiple cloning site obtained by hybridization of the following 2 oligonucleotides:

JCA082 (38 mer) (SEQ ID No. 19)
5'AATTGGCAGCTACATGGGATATCGGGCCCATCGATAAT 3'

JCA083 (34 mer) (SEQ ID No. 20)
5'ATTATCGATGGGCCCGATATCGGATGTAGCTGGC 3'

to give plasmid pPB206 (3548 bp).

Example 7: Isolation of the genomic RNA of the CDV strain Onderstepoort and cloning of the complementary DNA coding for the HA and F genes

The CDV strain Onderstepoort (Mitchell W. *et al*. J. Virol. Meth. 1987. **18**. 121-131) was cultured on MDCK (Madin-Darby canine kidney) cells in DMEM medium (Gibco). After purification of the virus, the genomic viral RNA was isolated using the guanidinium thiocyanate/phenol-chloroform extraction technique (Chomczynski P. and Sacchi N., Anal. Biochem. 1987. **162**. 156-159). Specific oligonucleotides (containing restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized in such a way as to cover completely the coding regions of the genes which were to be amplified (HA and F genes, respectively). The reverse transcription (RT) reaction and polymerase chain amplification (PCR) were performed according to the standard techniques (Sambrook J. *et al*. 1989). Each RT-PCR reaction was carried out with a pair of specific amplimers and taking as template the extracted viral genomic RNA. The amplified comple-

mentary DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with the restriction enzymes and cloned into the appropriate vector.

7.1. Construction of the CDV HA expression cassette (pPB208) (Figure 6)

The plasmid pCMVB (Clontech Ref. 6177-1) was digested with *EcoRI* and *NotI* to isolate an 818-bp EcoRI-NotI fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

An RT-PCR reaction was carried out with the genomic RNA of the CDV virus (strain Onderstepoort) and with the following oligonucleotides:

JCA084 (32 mer) (SEQ ID No. 21)

5'TTGCGGCCGCATGCTCCCCTACCAAGACAAGG 3'

JCA085 (28 mer) (SEQ ID No. 22)

5'TTGGTACCTTAACGGTTACATGAGAATC 3'

to obtain a 1837-bp PCR fragment containing the CDV HA gene. This fragment was digested with *NotI* and *KpnI* to isolate a 1826-bp NotI-KpnI fragment (fragment B).

The fragments A and B were ligated together with the vector pGEM-7Zf+ (Promega Cat # P2251), previously digested with *EcoRI* and *KpnI*, to give the plasmid pPB207 (5634 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:

PB088 (30 mer) (SEQ ID No. 23)

5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'

PB089 (32 mer) (SEQ ID No. 24)

5'TTGGGTACCGGATCCGAAAAAACCTCCCACAC 3'

to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene. This fragment was digested with *KpnI* to isolate a 233-bp KpnI-KpnI fragment. This fragment was then ligated with plasmid pPB207, previously digested with *KpnI*, to give plasmid pPB208 (5867 bp).

7.2. Construction of the CDV F expression cassette (pPB210) (Figure 7)

Plasmid pCMVB (Clontech Ref. 6177-1) was digested with *EcoRI* and *NotI* to isolate an 818-bp EcoRI-NotI fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

An RT-PCR reaction was carried out with the genomic RNA of the CDV virus (strain Onderstepoort) and with the following oligonucleotides:
JCA086 (34 mer) (SEQ ID No. 25)
5'TTGCGGCCGCATGCACAGGGGAATCCCCAAAAGC 3'
JCA087 (28 mer) (SEQ ID No. 26)
5'TTGGTACCTCAGAGTGATCTCACATAGG 3'
to obtain a 2011-bp PCR fragment containing the CDV F gene. This fragment was digested with *NotI* and *KpnI* to isolate a 2000-bp NotI-KpnI fragment (fragment B). The fragments A and B were ligated together with the vector pGEM-7Zf+ (Promega Ref. P2251), previously digested with *EcoRI* and *KpnI*, to give the plasmid pPB209 (5808 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:
PB088 (30 mer) (SEQ ID No. 23)
5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'
PB089 (32 mer) (SEQ ID No. 24)
5'TTGGGTACCGGATCCGAAAAAACCTCCCACAC 3'
to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene. This fragment was digested with *KpnI* to isolate a 233-bp KpnI-KpnI fragment. This fragment was then ligated with plasmid pPB209, previously digested with *KpnI*, to give plasmid pPB210 (6041 bp).

Example 8: Construction of the donor plasmid pPB213 for the insertion of the CDV HA expression cassette into th CHV ORF3 site (Figure 8)

Plasmid pPB208 (Example 7.1.) was digested with *ApaI* and *ClaI* to isolate a 2920-bp ApaI-ClaI fragment

containing the cassette for the expression of the CDV virus HA gene. This fragment was then ligated with plasmid pPB200 (Example 3), previously digested with *ApaI* and *ClaI*, to give plasmid pPB213 (7043 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF3 site.

Example 9: Construction of the donor plasmid pPB214 for the insertion of the CDV F expression cassette into the CHV ORF3 site (Figure 9)

Plasmid pPB210 (Example 7.2.) was digested with *ApaI* and *ClaI* to isolate a 3100-bp ApaI-ClaI fragment containing the cassette for the expression of the CDV virus F gene. This fragment was then ligated with plasmid pPB200 (Example 3), previously digested with *ApaI* and *ClaI*, to give plasmid pPB214 (7217 bp). This plasmid permits the insertion of the cassette for the expression of the CDV F gene into the CHV ORF3 site.

Example 10: Construction of the donor plasmid pPB215 for the insertion of the cassette for the expression of the rabies virus G gene into the CHV ORF3 site (Figures 10, 11 and 12)

Plasmid pPB199 (Example 3) was digested with *SpeI*, treated with alkaline phosphatase and then ligated with the multiple cloning site obtained by hybridization of the following 2 oligonucleotides:
JCA088 (39 mer) (SEQ ID No. 27)
5'CTAGTCCAGCAAGGTGTCGACGGATCGATATCGGGCCCA 3'
JCA089 (39 mer) (SEQ ID No. 28)
5'CTAGTGGGCCCGATATCGATCCGTCGACACCTTGCTGGA 3'
to give plasmid pPB200' (4135 bp) (Figure 10).

Plasmid pCMVB (Clontech Ref. 6177-1) was digested with *EcoRI* and *NotI* to isolate an 818-bp EcoRI-NotI fragment containing the promoter region of the human cytomegalovirus immediate-early gene (fragment A).

According to the technical procedures already described for the CDV virus (Example 7), the RNA of the

rabies virus ERA strain was extracted and purified from a culture of rabies virus-infected Vero cells. An RT-PCR reaction was then carried out (see Example 7) with the genomic RNA of the rabies virus (strain ERA) and with the following oligonucleotides:

JCA090 (31 mer) (SEQ ID No. 29)

5'TTGCGGCCGCATGGTTCCTCAGGCTCTCCTG 3'

JCA091 (31 mer) (SEQ ID No. 30)

5'TTGGTACCTCACAGTCTGGTCTCACCCCCAC 3'

to obtain a 1597-bp PCR fragment containing the rabies virus G gene (Patents FR-A-2,515,685 and EP-A-162,757). This fragment was digested with *Not*I and *Kpn*I to isolate a 1586-bp NotI-KpnI fragment (fragment B). The fragments A and B were ligated together with the vector pSP73 (Promega Ref. P2221), previously digested with *EcoR*I and *Kpn*I, to give the plasmid pPB211 (4852 bp).

A PCR reaction was carried out with plasmid pCMVB and with the following oligonucleotides:

PB088 (30 mer) (SEQ ID No. 23)

5'TTGGGTACCGCCTCGACTCTAGGCGGCCGC 3'

PB089 (32 mer) (SEQ ID No. 24)

5'TTGGGTACCGGATCCGAAAAAACCTCCCACAC 3'

to obtain a 244-bp PCR fragment containing the polyadenylation signal of the SV40 virus early gene. This fragment was digested with *Kpn*I to isolate a 233-bp KpnI-KpnI fragment. This fragment was then ligated with plasmid pPB211, previously digested with *Kpn*I, to give plasmid pPB212 (5085 bp) (Figure 11). Plasmid pPB212 was digested with *EcoRV* and *Sal*I to isolate a 2664-bp EcoRV-SalI fragment containing the cassette for the expression of the rabies virus G gene. This fragment was then ligated with plasmid pPB200' (see above), previously digested with *EcoRV* and *Sal*I, to give plasmid pPB215 (6790 bp) (Figure 12).

Example 11: Construction of the donor plasmid pPB216 for the insertion of the CDV HA expression cassette into the CHV ORF5 site (Figure 13)

Plasmid pPB208 (Example 7.1.) was digested with *Sma*I and *Apa*I to isolate a 2909-bp SmaI-ApaI fragment containing the cassette for the expression of the CDV HA gene. This fragment was then ligated with plasmid pPB202 (Example 4), previously digested with *EcoR*V and *Apa*I, to give plasmid pPB216 (6291 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV ORF5 site.

Example 12: Construction of the donor plasmid pPB217 for the insertion of the CDV HA expression cassette into the CHV TK site (Figure 14)

Plasmid pPB208 (Example 7.1.) was digested with *Apa*I and *Cla*I to isolate a 2920-bp fragment containing the cassette for the expression of the CDV HA gene. This fragment was ligated with plasmid pPB204 (Example 5), previously digested with *Apa*I and *Cla*I, to give plasmid pPB217 (6316 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the CHV TK site.

Example 13: Construction of the donor plasmid pPB218 for the insertion of the CDV HA expression cassette into the site situated between the CHV ORF19 and CHV ORF22 genes (Figure 15)

Plasmid pPB208 (Example 7.1.) was digested with *Apa*I and *Cla*I to isolate a 2920-bp fragment containing the cassette for the expression of the CDV HA gene. This fragment was ligated with plasmid pPB206 (Example 6), previously digested with *Apa*I and *Cla*I, to give plasmid pPB218 (6462 bp). This plasmid permits the insertion of the cassette for the expression of the CDV HA gene into the site situated between the CHV ORF19 and CHV ORF22 genes.

Example 14: Isolation of the recombinant virus vCHV01 containing th cassette for the expression of the CDV HA gene in the CHV ORF3 site.

Plasmid pPB213 (see Example 8) was linearized by digestion with *Hin*dIII, extracted with a phenol-chloroform mixture, precipitated with absolute ethanol and then taken up in sterile water.

MDCK cells forming a well-established cell lawn in a Petri dish (Corning 4.5 cm in diameter) were then transfected with the following mixture:
1 μg of linearized plasmid pPB213 + 5 μg of CHV viral DNA in 300 μl of MEM medium and 100 μg of LipofectAMINE (Gibco-BRL Cat# 18324-012) diluted in 300 μl of medium (final volume of the mixture = 600 μl). These 600 μl were then diluted in 3 ml (final volume) of MEM medium and spread over 3 × 10^6 MDCK cells. The mixture was left in contact with the cells for 5 hours, then removed and replaced by 5 ml of culture medium. The cells were then left in culture for 24 hours at +37°C. After 24 hours to 48 hours of culture, 1 ml of culture supernatant was harvested, and several dilutions of this supernatant were used to infect other MDCK cells (cultured in Petri dishes (Corning 4.5 cm in diameter)) so as to obtain isolated plaques, each dish being infected with 1 ml of a dilution of the initial supernatant. After contact for 1 hour at 37°C, the infection medium was removed and replaced by 5 ml of MEM medium containing 1% of agarose, kept supercooled at 42°C. When the agarose had solidified, the dishes were incubated for 48 hours at 37°C in a CO_2 incubator until plaques were seen. The agarose layer was then removed, and a transfer of the viral plaques was carried out onto a sterile nitrocellulose membrane of the same diameter as the Petri dish used for culturing. This membrane was itself transferred onto another nitrocellulose membrane so as to obtain an inverted "copy" of the first transfer. The plaques transferred onto this second copy were then hybridized, according to the standard techniques known to a person skilled in the art, with a 1842-bp NotI-

NotI fragment of the CDV HA gene, obtained by digestion of plasmid pPB208 (Example 7.1.), labelled with digoxigenin (DNA Labelling kit, Boehringer Mannheim, CAT # 1175033). After hybridization, washes and contacting with the visualization substrate, the nitrocellulose membrane was placed in contact with an autoradiographic film. The images of positive hybridization on this membrane indicated which plaques were the ones which contained recombinant CHV viruses which had inserted the CDV HA cassette. The plaques corresponding to these positive plaques were cut out under sterile conditions from the first nitrocellulose membrane, placed in an Eppendorf tube containing 0.5 ml of MEM medium and sonicated to release the virions from the membrane. The medium contained in the Eppendorf tube was then diluted in MEM medium, and the dilutions thereby obtained were used to infect further cultures of MDCK cells. A 100% pure recombinant virus containing the HCMV-IE/CDV HA/polyA cassette inserted into the ORF3 site was thereby isolated after 3 cycles of purification, and was called vCHV01. The homology of the recombination was verified by PCR using oligonucleotides situated on each side of the insertion site. The absence of reorganization on the genome of the recombinant virus vCHV01, other than in the recombination region, was verified by the Southern blot technique.

Example 15: Isolation of recombinant CHV viruses expressing various foreign genes

According to the procedure described in Example 14, the construction of different recombinant CHV viruses is carried out using the donor plasmids described in Examples 9 to 13.

Example 16: Preparation of the vaccines

To prepare a vaccine, the recombinant viruses obtained in Examples 14 and 15 are cultured on MDCK cells. Harvesting of the recombinant virus takes place

when the cytopathic effect is complete. The lysed cells and the culture supernatant are harvested. After clarification of the cell lysate to remove cell debris, the viral solution is titrated. The viral solution is then diluted in a stabilizing solution for lyophilization, distributed on the basis of one vaccinal dose (10^2 CCID50 to 10^7 CCID50) per vial and lastly lyophilized. The viral solution can then be frozen if necessary.

Example 17: Vaccination methods

According to the preferred mode of vaccination, the vaccine obtained according to the invention is redissolved or thawed, and then administered via the parenteral or mucosal route, but preferably via the mucosal, in particular the oral and/or nasal, route. The vaccinal dose will preferably be between 10^2 CCID50 and 10^7 CCID50. Preferably, the dose for the parenteral route will be between 10^4 CCID50 and 10^7 CCID50, and for the oral and/or nasal route, between 10^2 CCID50 and 10^5 CCID50. As defined, the vaccine is effective in general after a single administration via the oral and/or nasal route. However, repeated administrations may be necessary.